

Original Paper

MCP-1 Induced Protein Promotes Adipogenesis via Oxidative Stress, Endoplasmic Reticulum Stress and Autophagy

Craig Younce and Pappachan Kolattukudy

Burnett School of Biomedical Sciences, University of Central Florida, Bldg. 20, Rm 136, Orlando, FL

Key Words

Monocyte Chemotactic Protein-1 • Endoplasmic Reticulum Stress • Oxidative Stress • Adipogenesis • MCP-1-induced Protein • Autophagy

Abstract

Obesity involves inflammation. MCP-1, an inflammatory chemokine, and MCP-1-induced protein (MCPIP) are known to induce adipogenesis that causes increase in the number of adipocytes. Here we elucidate the intermediate processes through which MCPIP induces adipogenesis. Forced expression of MCPIP in 3T3-L1 preadipocytes caused increased reactive oxygen/nitrogen species (ROS/RNS) production and inducible-nitric oxide synthase (iNOS) expression, endoplasmic reticulum stress (ER), as indicated by expression of ER chaperones and protein disulfide isomerase, and autophagy as indicated by expression of beclin-1 and cleavage of LC3. Treatment of ROS inhibitor, apocynin attenuated MCPIP induction of adipogenesis as measured by the induction of transcription factors involved in adipogenesis, adipocyte markers and lipid droplet accumulation. Inhibition of ER stress with taurursodeoxycholate or knockdown of inositol requiring enzyme 1 (IRE1) inhibited MCPIP induced autophagy and adipogenesis. Preadipocytes in adipogenesis-inducing cocktail manifested ER stress and autophagy. Knockdown of MCPIP attenuated these effects. MCPIP induced p38 activation and p38 inhibitor, SB203580, attenuated MCPIP-induced adipogenesis. Inhibition of autophagy by specific inhibitors or knockdown of beclin-1 attenuated adipogenesis. These results demonstrate that MCPIP mediates adipogenesis via ROS/RNS production that causes ER stress that leads to autophagy required for adipocyte differentiation, that plays an important role in obesity.

Copyright © 2012 S. Karger AG, Basel

Introduction

Obesity is associated with an increased risk for a set of health complications known as metabolic syndrome which includes type 2 diabetes [1]. Excess fat in healthy individuals is stored in adipocytes and low amounts of triacylglycerol are maintained in nonadipocytes. In obese patients, the amount of fat that is capable of being stored in the adipocyte tissue can be exceeded resulting in abnormal lipid accumulation in hepatic, pancreatic, and muscular tissues. Abnormal lipid accumulation in these tissue types eventually results in increased dysfunction of those tissues and thus may contribute to the development of type 2 diabetes [1].

An increase in adipose tissue mass is characteristic of obesity [2]. This increase consists of an increase in the number of fat cells as well as an increase in the size of the fat cells. The process whereby fibroblast-like preadipocytes differentiate into mature adipocytes is known as adipogenesis. Adipogenesis is a process of differentiation systematically controlled by a set of well characterized transcription factors. Two transcription factor families have emerged as the key determinants of terminal adipocyte differentiation: the C/EBP α , - β and - δ , and PPAR γ [3, 4]. As cells undergo differentiation process in response to adipogenic signals, the initial event is the rapid induction of C/EBP β and - δ expression [5]. A role for C/EBP β and - δ in the induction of PPAR γ 2, a key regulator of adipogenesis, has been reported [6, 7]. During the later stage of differentiation, C/EBP α expression rises immediately after PPAR γ 2 expression and several studies have demonstrated that PPAR γ 2 and C/EBP α can regulate each other's expression [8, 9].

In recent years it has become increasingly clear that obesity involves a low-grade systemic inflammatory condition [10]. Both adipocytes and macrophages are sources of cytokine production [11]. Monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2 have been implicated in obesity and type 2 diabetes. Obesity development caused by high fat diet feeding is attenuated by the absence of CCR2 [12]. How CCR2 contributes to obesity is unknown. Monocyte chemoattractant protein-1 (MCP-1) has been shown to be produced in preadipocytes stimulated with the adipogenesis inducing cocktail, dexamethasone, 3-isobutyl-1-methylxanthine, insulin (DMI) [13]. Moreover, MCP-1 was recently found to contribute to adipocyte differentiation that can lead to an increase in adipose tissue mass [14]. MCP-1 induced adipogenesis was found to occur via induction of a novel zinc-finger protein, MCP-1-induced protein (MCPIP) resulting from the signaling events triggered by binding to CCR2 [12, 14]. How MCPIP mediates adipogenesis remains unknown.

In this report we provide evidence that in 3T3-L1 cells, MCPIP induces adipogenesis via induction of reactive oxygen/nitrogen species (ROS/RNS) that leads to endoplasmic reticulum (ER) stress. ER stress results in the induction of autophagy that is involved in adipocyte differentiation. MCPIP induced ROS/RNS production was found to occur early and lead to the induction of the adipogenic factors C/EBP β , C/EBP δ , C/EBP α and PPAR γ . Our results indicate that MCPIP-induced ER stress and autophagy occurred after the initial stages of adipogenesis and contributed only to the induction of C/EBP α and PPAR γ . Thus, our results elucidate the nature of the processes through which MCPIP mediates adipogenesis providing potential therapeutic targets for the prevention and treatment of obesity and type-2 diabetes.

Materials and Methods

Cell Culture

The 3T3-L1 fibroblast cell line was obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium with 1% penicillin/streptomycin and 10% fetal bovine serum until experimentation. Adipogenesis was induced using DMI were indicated. Otherwise cells were transfected with MCPIP. Construction of the MCPIP-GFP expression plasmid has been described

previously [15]. All cells were transfected using FuGENE HD (Roche Applied Science). Cells were treated with or without 50 $\mu\text{mol/L}$ 3'-methyladenine (3'MA); 1 $\mu\text{mol/L}$ LY294002; 100 $\mu\text{mol/L}$ tauroursodeoxycholate (TUDC); or 20 $\mu\text{mol/L}$ apocynin starting 3hr prior to transfection.

siRNA Treatment

Cells were treated with 100nmol/L of a chemically synthesized siRNA targeted for MCPiP, beclin-1, or inositol requiring enzyme 1 (IRE1) (Ambion) or with 100nmol/L non-specific siRNA (Ambion) using Dharmafect transfection reagent 12hrs prior to transfection with MCPiP-GFP.

Oil Red-O

3T3-L1 cells were fixed with 10% formalin for 30 min at room temperature 8 days after experimental treatment. Cells were then washed with 60% isopropyl alcohol followed by treatment with Oil Red-O (2.1 mg/ml) for 20 min at room temperature. Samples were then washed four times with H_2O . Oil Red-O was evaluated by measuring total objects stained, area of plate stained, and total intensity of stained area. All three methods yielded similar results. After examining the plates microscopically, they were treated with 100% isopropyl alcohol to extract Oil Red-O. The solution was then measured for absorbance at 520 nm.

ROS/RNS Measurements

ROS/RNS production was evaluated fluorometrically using Dihydrorhodamine 123 (DHR123). Briefly, starting 12hr after transfection with MCPiP-GFP or GFP alone, cells were treated with 1 $\mu\text{mol/L}$ DHR123 for 30min. at 37 C and 5% CO_2 . Cells were then washed 3x with 1x PBS. 5×10^5 cells were plated on a 96 well plate and were subjected to fluorometric analysis (Excitation: 550nm; Emission: 590nm).

RT-PCR

Total RNA was isolated from treated fibroblasts using TRIzol (Invitrogen). First-strand cDNA was synthesized using 1 μg of total RNA (DNase-treated) using the RT cDNA synthesis kit (High Capacity, Applied Biosystems). β -Actin served as internal control.

Immunoblot Analysis

3T3-L1 were treated with cell lysis buffer (20% glycerol, 0.1% Triton X-100, 8% 0.5 M EDTA, and 1% 1 M dithiothreitol), and protein samples were collected and subjected to immunoblot using polyclonal antibodies specific for adiponectin (1:3000, Abcam), lipoprotein lipase (LPL) (1:2000, Abcam), glucose regulated protein 78 (GRP78) (1:2000, Santa Cruz), protein disulfide isomerase (PDI) (1:2000, Santa Cruz), iNOS (1:2000, Upstate), LC3 (1:1000, Cell Signaling), or Beclin-1 (1:500, Santa Cruz). Immunoblots were quantified as a ratio over β -actin expression.

Statistical Analysis

The experimental data was analyzed by using Graphpad statistical software (SPSS Inc.). All values are presented as mean + SEM. Results were compared between groups by ANOVA analysis followed by turkey post tests. Differences were considered significant at a p value of <0.05.

Results

MCPiP induces adipogenesis via oxidative stress

Oxidative stress can induce adipogenesis [16-18]. To test whether MCPiP induced oxidative stress is involved in adipogenesis, we transfected 3T3-L1 cells with MCPiP-GFP or GFP alone (Fig. 1A) and measured total ROS production using dihydrorhodamine 123. MCPiP induced ROS production in a time dependent manner with induction starting at 12hr after transfection and reaching maxima at 24hr (Fig. 1B). To determine if MCPiP-induced oxidative stress is not limited to ROS production, we collected cell lysate from 3T3-L1 cells transfected with MCPiP-GFP or GFP alone and subjected them to immunoblot analysis for inducible nitric oxide synthase (iNOS) expression levels (Fig. 1C). iNOS expression was found to be induced in MCPiP-GFP expressing cells.

To test whether the induction of oxidative stress is involved in the mediation of the expression of the key adipogenic transcription factors C/EBP β , C/EBP δ , C/EBP α and PPAR γ ,

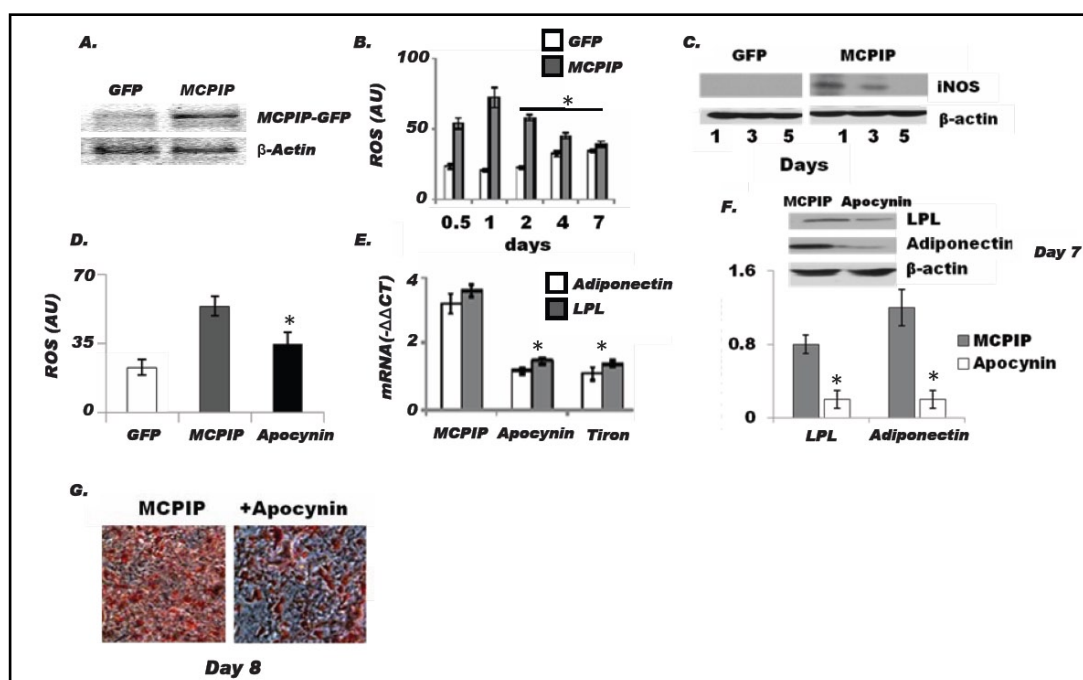


Fig. 1. MCPIP-induced adipogenesis is mediated via iNOS and NADPH oxidase induced ROS/RNS production: (A), 3T3-L1 preadipocytes were transfected with MCPIP-GFP or GFP alone. 1 day post transfection, 20μg of cell lysate was taken and immunoblot was performed using antibody against MCPIP. (B), ROS/RNS was measured at 0.5, 1, 2, 4, and 7 days post transfection by treating MCPIP-GFP or GFP expressing cells with 1μM DHR123 for 30min. Cells were then examined microscopically and fluorometrically (excitation 550nm; emission 590nm; $p < 0.05$). (C), 3T3-L1 preadipocytes were transfected with MCPIP-GFP or GFP alone. At 1, 3 and 5 days post transfection 20μg of cell lysate was taken and immunoblot was performed using antibody against iNOS. (D-G), 3T3-L1 preadipocytes were transfected with MCPIP-GFP and with/without apocynin. (D), 1 day post transfection, ROS/RNS was measured with 1μM DHR123 for 30min. Cells were then examined fluorometrically (excitation 550nm; emission 590nm; $p < 0.05$) (E and F), RNA and cell lysate were collected 7 days after transfection. (D). Transcript levels of the adipocyte markers, adiponectin and LPL, were measured using RT-PCR; β-actin served as a control; $-\Delta\Delta Ct$ values of MCPIP-GFP/GFP are displayed ($p < 0.05$). (F), protein levels of the adipocyte markers, adiponectin and LPL, were measured using immunoblot analysis. Results were quantified and normalized to β-actin ($p < 0.05$). (G), Cells were stained with Oil Red-O 8 days after transfection. All experiments were repeated three times.

3T3-L1 cells were treated with apocynin prior to transfection with MCPIP-GFP and were evaluated using real time RT-PCR for C/EBPβ, C/EBPδ, C/EBPα and PPARγ (Fig. 2). Apocynin attenuated MCPIP-induction of C/EBPβ, C/EBPδ, C/EBPα and PPARγ. To confirm that apocynin attenuated MCPIP-induced ROS production, we measured total ROS production using dihydrorhodamine 123. As expected, apocynin attenuated MCPIP-induced ROS expression (Fig. 1D).

To test whether MCPIP-induced oxidative stress is involved in adipogenesis, we determined the effect of ROS inhibitor apocynin on adipogenesis (Fig. 1E, F). Apocynin treatment attenuated MCPIP-induced expression of the adipocyte markers adiponectin and LPL at both the transcript and protein levels and inhibited MCPIP-induced oil droplet accumulation as detected by Oil-Red O (Fig. 1G). These results demonstrate that MCPIP-induced oxidative stress plays a key role in MCPIP-induced adipogenesis.

MCPIP-induced adipogenesis involves ER stress

Oxidative stress is known to lead to ER stress [19, 20]. To test if MCPIP-induced adipogenesis involves an ER stress response, we examined cell lysate from 3T3-L1 preadipocytes expressing MCPIP-GFP or GFP for the ER chaperone proteins GRP78 and PDI by immunoblot analysis. Cells expressing MCPIP-GFP showed elevated expression of

Fig. 2. MCPIP-induced ROS mediates an early response in MCPIP-induced adipogenesis by inducing both early and late adipogenic transcription factors: 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with or without apocynin. RNA was collected 1, 3, and 5 days after transfection. Transcript levels of the adipogenesis markers C/EBP β (A), C/EBP δ (B), C/EBP α (C), and PPAR γ (D) were measured using real time RT-PCR (normalized to β -actin; $-\Delta\Delta C_t$ values of MCPIP-GFP/GFP are displayed).

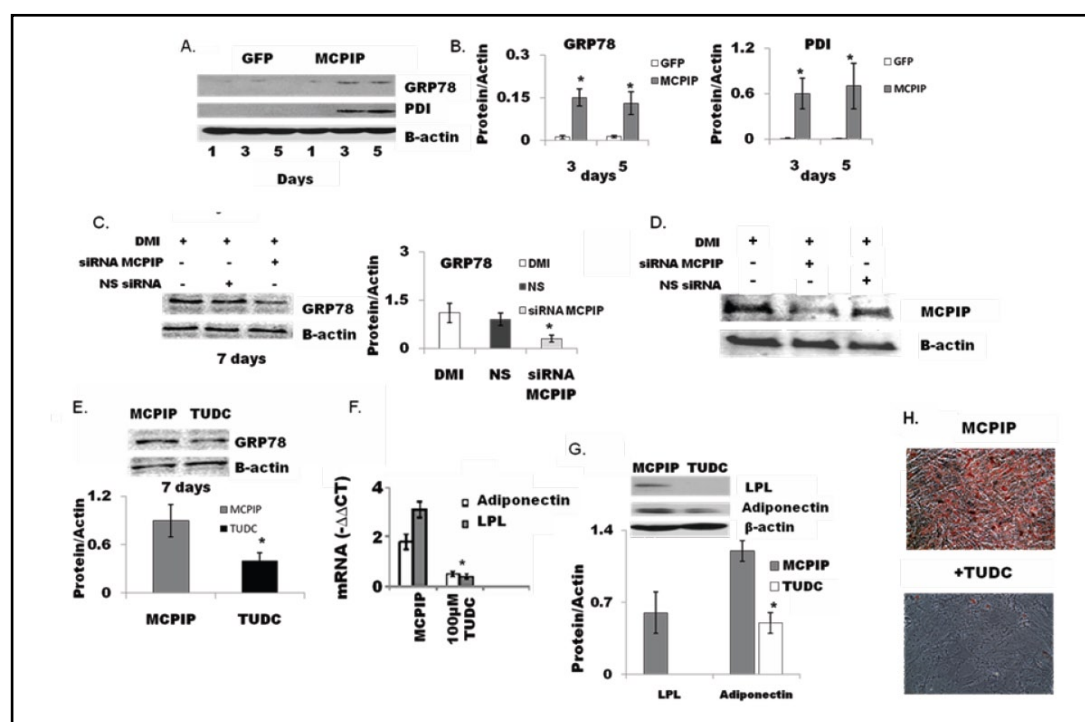
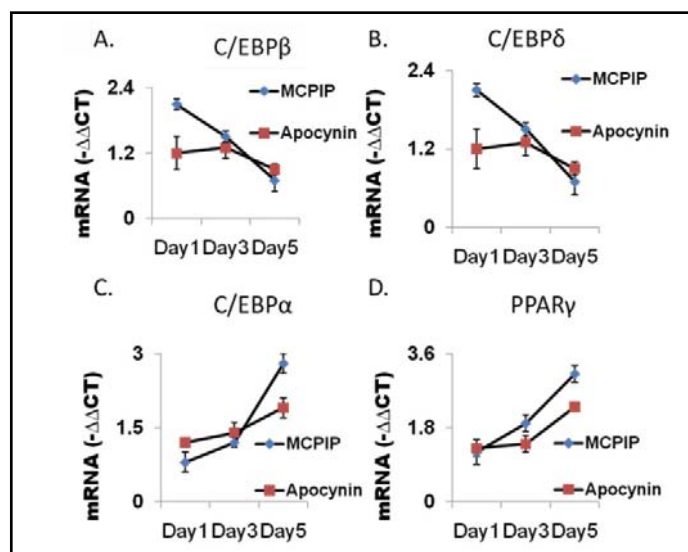


Fig. 3. MCPIP-induced adipogenesis is mediated via ER stress: (A), 3T3-L1 preadipocytes were transfected with MCPIP-GFP or GFP alone. At 1, 3 and 5 days post transfection 20 μ g of cell lysate was taken and immunoblot was performed using antibody against GRP78 and PDI. (B), Results were quantified and normalized to β -actin ($p < 0.05$). (C), 3T3-L1 preadipocytes were treated with DMI and with or without 100nmol/L siRNA specific to MCPIP or with non-specific (NS) siRNA. At 7 days, cell lysate was taken and immunoblot was performed using antibody specific to GRP78 or (D), MCPIP. Results were quantified and normalized to β -actin ($p < 0.05$). (E), 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with or without 100 μ M TUDC. At 7 days cell lysate was taken and immunoblot was performed using antibody specific to GRP78 (E-F), RNA and cell lysate were collected 7 days after transfection. (F), Transcript levels of the adipocyte markers, adiponectin and LPL, were measured using RT-PCR; β -actin served as a control; $-\Delta\Delta C_t$ values of MCPIP-GFP/GFP are displayed. Results were normalized to β -actin ($p < 0.05$). (G), protein levels of the adipocyte markers, adiponectin and LPL, were measured using immunoblot analysis. Results were quantified and normalized to β -actin ($p < 0.05$). (H), Cells were stained with Oil Red-O 8 days after transfection. All experiments were repeated three times.

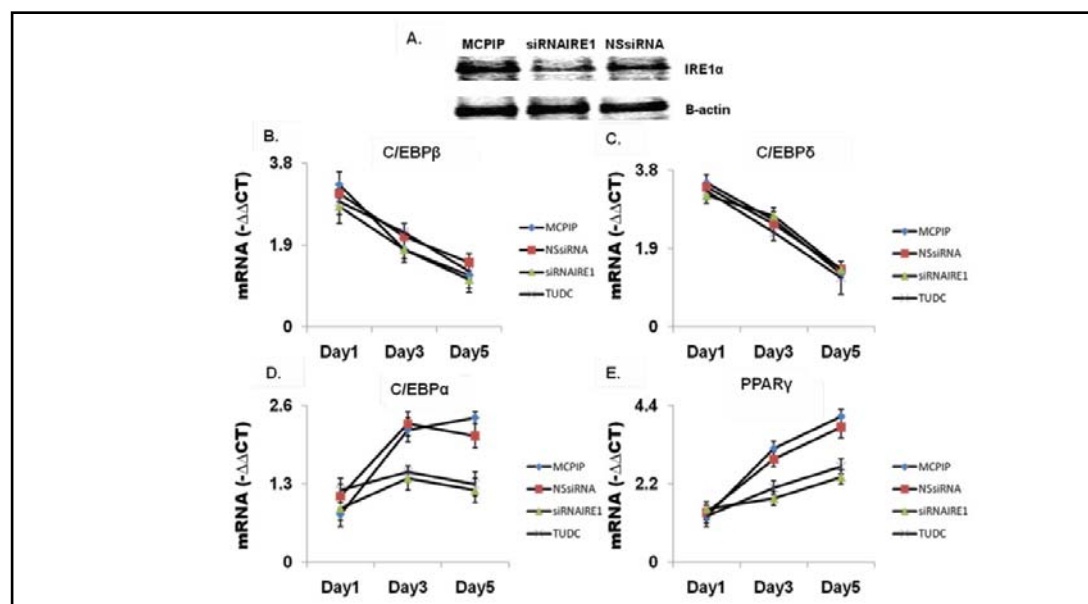


Fig. 4. MCPiP-induced ER stress mediates induction of the adipogenic transcription factors C/EBP α and PPAR γ but not C/EBP β or C/EBP δ : (A), 3T3-L1 preadipocytes were transfected with MCPiP-GFP and treated with/ without 100nM siRNA specific for IRE1 or non-specific siRNA (NS siRNA). 1 day post transfection, 20 μ g of cell lysate was taken and immunoblot was performed using antibody against IRE1. (B), 3T3-L1 preadipocytes were transfected with MCPiP-GFP and treated with/ without 100nM siRNA specific for IRE1 or non-specific siRNA (NS siRNA) or with/without 100 μ M TUDC. RNA was collected 1, 3, and 5 days after transfection. Transcript levels of the adipogenesis markers C/EBP β (A), C/EBP δ (B), C/EBP α (C), and PPAR γ (D) were measured using real time RT-PCR (normalized to β -actin; $-\Delta\Delta C_t$ values of MCPiP-GFP/GFP are displayed).

GRP78 and PDI when compared to GFP controls with significant levels detected at 3 and 5 days after transfection (Fig. 3A, B). DMI-induced adipogenesis is known to involve ER stress signaling [21]. To determine if MCPiP is critically involved in DMI-induced adipogenesis, 3T3-L1 preadipocytes were treated with DMI and MCPiP expression was knocked down using specific siRNA. We found that MCPiP knockdown (Fig. 3D) resulted in a reduction of DMI-induced GRP78 protein expression (Fig. 3C). To test whether MCPiP-induced ER stress contributes directly to adipogenesis, we treated 3T3-L1 preadipocytes with the chemical chaperone TUDC, known to selectively inhibit ER stress [22], prior to transfection with MCPiP-GFP. TUDC treatment inhibited MCPiP-induced expression of the ER stress marker GRP78 as detected by immunoblot analysis (Fig. 3E), reduced expression of adiponectin and LPL (Fig. 3F, G), and attenuated the accumulation of lipid droplets as detected by Oil Red-O (Fig. 3H). These results demonstrate that ER stress induced by MCPiP is involved in MCPiP-induced adipogenesis.

Since MCPiP-induced ER stress occurs during the medial and later stages of MCPiP-induced adipogenesis, it is plausible that MCPiP-induced ER stress regulates the expression of only a subset of the key adipogenic transcription factors such as C/EBP α and PPAR γ that are normally expressed later during adipogenesis. To test for this possibility, we determined the effect of inhibition of ER stress by knocking down IRE with specific siRNA (Fig. 4A) or with the ER stress inhibitor, TUDC, upon MCPiP-induced expression of C/EBP β , C/EBP δ , C/EBP α and PPAR γ (Fig. 4B-E). We found that inhibition of ER stress caused attenuation of MCPiP-induced expression of C/EBP α and PPAR γ but not C/EBP β or C/EBP δ . These results strongly suggest that MCPiP-induced ER stress plays a key role during the medial to later stages of MCPiP-induced adipogenesis.

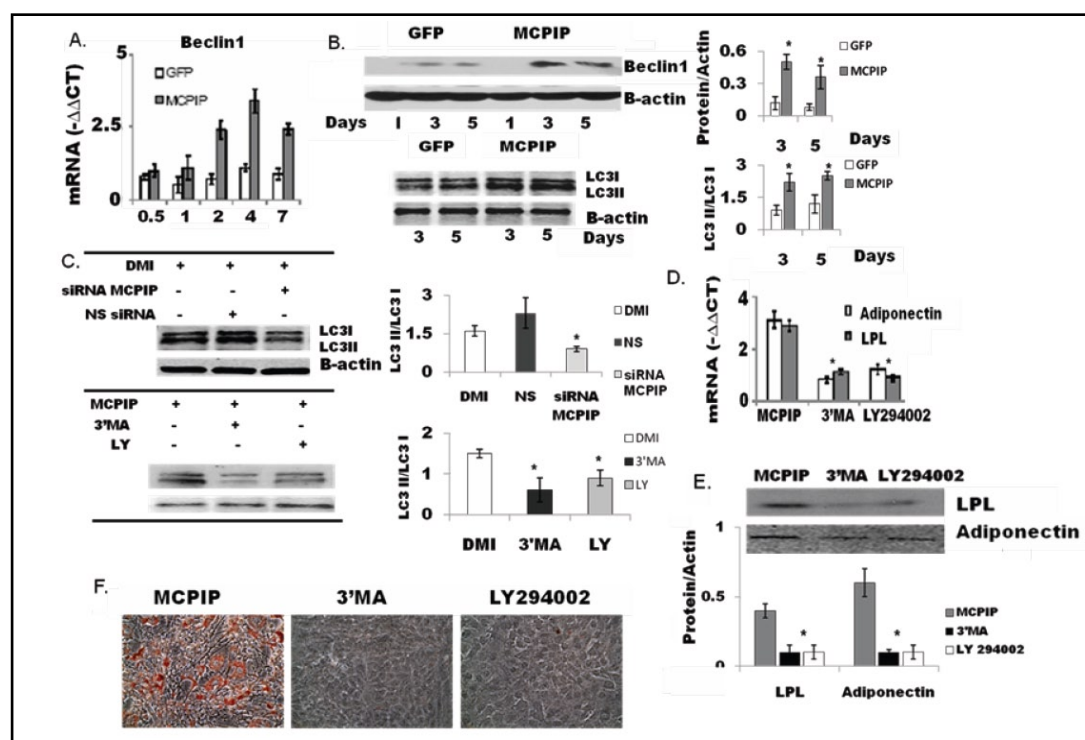


Fig. 5. MCPiP-induced adipogenesis is mediated via autophagy: 3T3-L1 preadipocytes were transfected with MCPiP-GFP or GFP alone. (A), RNA was collected 0.5, 1, 2, 4 days and 7 days after transfection. Transcript levels of the autophagy marker, beclin-1, were measured using RT-PCR; β -actin served as a control; $-\Delta\Delta C_t$ values of MCPiP-GFP/GFP are displayed. (B), At 1, 3 and 5 days post transfection, 20 μ g of cell lysate was taken and immunoblot was performed using antibody specific to (top) beclin-1 or (bottom) LC3 I and II. Results were normalized to β -actin and quantified as a ratio of LC3II product/ LC3I ($p < 0.05$). (C, top), 3T3-L1 preadipocytes were treated with DMI and with or without 100nmol/L siRNA specific to MCPiP or with non-specific (NS) siRNA. At 5 days post transfection 20 μ g of cell lysate was taken and immunoblot was performed using antibody specific to LC3 I and II. Results were normalized to β -actin and quantified as a ratio of LC3II product/ LC3I ($p < 0.05$). (C, bottom), 3T3-L1 preadipocytes were transfected with MCPiP-GFP or GFP alone and with/without 50iM 3'MA or 1 μ M LY294002. At 5 days post transfection 20 μ g of cell lysate was taken and immunoblot was performed using antibody specific to LC3 I and II. Results were normalized to β -actin and quantified as a ratio of LC3II product/ LC3I ($p < 0.05$). (D-E), RNA and cell lysate were collected 7 days after transfection. (D), Transcript levels of the adipocyte markers, adiponectin and LPL, were measured using RT-PCR; β -actin served as a control; $-\Delta\Delta C_t$ values of MCPiP-GFP/GFP are displayed. Results were normalized to β -actin ($p < 0.05$). (E), protein levels of the adipocyte markers, adiponectin and LPL, were measured using immunoblot analysis. Results were normalized to β -actin and quantified as a ratio of LC3II product/ LC3I ($p < 0.05$). (F), Cells were stained with Oil Red-O 8 days after transfection. All experiments were repeated three times.

MCPiP-induced adipogenesis is mediated via autophagy

ER stress is known to cause autophagy [23]. A role for autophagy in adipogenesis has also been implicated [24, 25]. To test if MCPiP-induced adipogenesis is mediated via autophagy, we transfected 3T3-L1 preadipocytes with MCPiP-GFP or GFP alone and measured the transcript levels of the autophagy marker, beclin-1. Elevation of beclin transcripts began on day 2, reached maxima on day 4, and began to declining on day 7 after transfection (Fig. 5A). Immunoblot analysis revealed that beclin-1 protein level and LC3 cleavage also increased beginning on day 3 and remained at high levels until 5 days (Fig. 5B). To determine if MCPiP is required for the induction of autophagy in adipogenesis, 3T3-L1 preadipocytes were treated with DMI and MCPiP was knocked down using specific siRNA. We found that knockdown

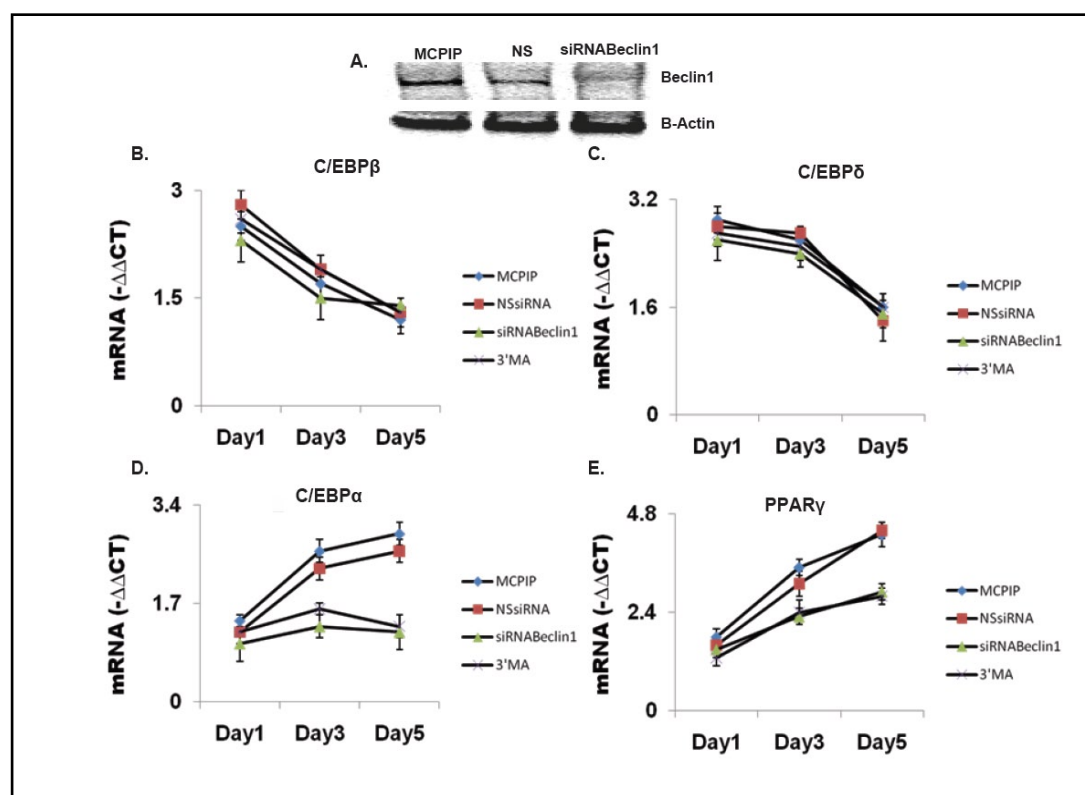


Fig. 6. MCPIP-induced autophagy mediates induction of the adipogenic transcription factors C/EBP α and PPAR γ but not C/EBP β or C/EBP δ : 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with/ without 100nM siRNA specific for Beclin1 or non-specific siRNA (NS siRNA) or with/without 50 μ M 3'MA. (A), 1 day post transfection, 20 μ g of cell lysate was taken and immunoblot was performed using antibody against Beclin1. (B-E), RNA was collected 1, 3, and 5 days after transfection. Transcript levels of the adipogenesis markers C/EBP β (B), C/EBP δ (C), C/EBP α (D), and PPAR γ (E) were measured using real time RT-PCR (normalized to β -actin; $-\Delta\Delta Ct$ values of MCPIP-GFP/GFP are displayed).

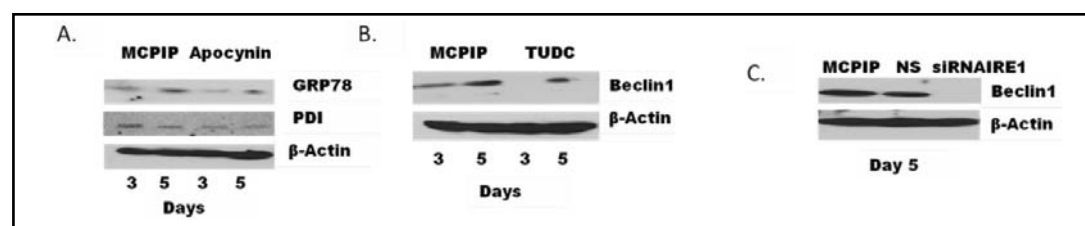


Fig. 7. MCPIP-induced ROS results in ER stress that causes autophagy: (A), 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with or without 50 μ M apocynin. At 3 and 5 days post transfection, 20 μ g of cell lysate was taken and immunoblot was performed using antibody against GRP78 and PDI. (B), 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with or without 100 μ M TUDC. At 3 and 5 days post transfection, 20 μ g of cell lysate was taken and immunoblot was performed using antibody against beclin-1. (C), 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with/ without 100nM siRNA specific for IRE1 or non-specific siRNA (NS siRNA). At 5 days post transfection, 20 μ g of cell lysate was taken and immunoblot was performed using antibody against beclin-1.

of MCPIP (Fig. 3D) attenuated DMI-induced cleavage of LC3, indicative of a reduction in autophagy (Fig. 5C, top). Autophagy inhibitors, 3'-methyladenine and LY294002 attenuated MCPIP-induced cleavage of LC3 as indicated by immunoblot analysis (Fig. 5C, lower). The autophagy inhibitors 3'MA or LY294002 inhibited MCPIP-induced expression of adiponectin and LPL at both transcript and protein levels (Fig. 5D, E). These autophagy inhibitors also

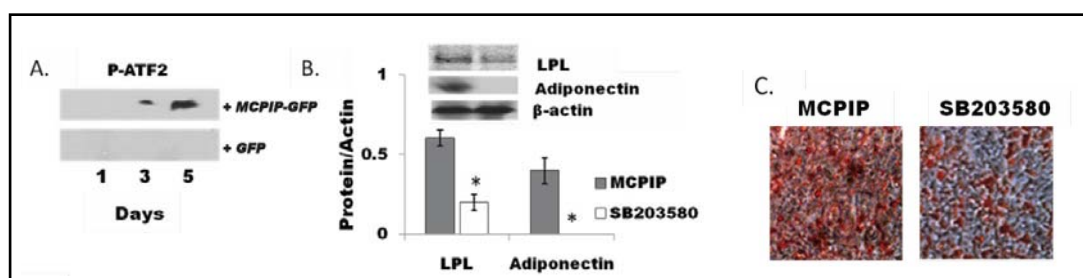


Fig. 8. MCPIP-induced adipogenesis is mediated via p38 activation: (A), p38 was immunoprecipitated from the cell lysate of MCPIP expressing 3T3-L1 preadipocytes. Immunoprecipitated p38 was combined with ATF2, a known target of activated p38 and ATF2 was tested for phosphorylation using immunoblot analysis. (B-C), 3T3-L1 preadipocytes were transfected with MCPIP-GFP and treated with or without SB203580. (B), Protein levels of the adipocyte markers, adiponectin and LPL, were measured using immunoblot analysis. Results were quantified and normalized to β -actin ($p < 0.05$). (C), Cells were stained with Oil Red-O 8 days after transfection. All experiments were repeated three times.

severely inhibited lipid accumulation as detected by Oil Red-O staining of the lipid bodies. (Fig. 5F). This indicated that MCPIP-induced lipid accumulation is mediated via induction of autophagy.

That beclin-1 expression is observed in the medial to later stages of MCPIP induced adipogenesis suggests that it might be involved in the regulation of expression of the transcription factors known to be expressed at these stages. In fact, we found that knockdown of beclin-1 (Fig. 6A) had no effect on the induction of C/EBP β and C/EBP δ that are induced during the early stages of adipogenesis. (Fig. 6), but attenuated the induction of transcript levels of C/EBP α and PPAR γ that are induced in the late stages of induction of adipogenesis (Fig. 6). Inhibition of autophagy by 3' MA caused similar effects (Fig. 6). These results indicate that autophagy is induced after the initial induction of C/EBP β and C/EBP δ but prior to the later induction of C/EBP α and PPAR γ . Thus, MCPIP-induced adipogenesis requires autophagy to regulate the later adipogenic factors necessary for development of mature adipocytes.

MCPIP-induced adipogenesis is mediated via ROS that causes ER stress that leads to autophagy

To test whether MCPIP-induced oxidative stress causes ER stress that leads to autophagy involved in adipogenesis, we tested whether inhibition of each step blocked the postulated subsequent steps. Inhibition of ROS with apocynin resulted in reduced expression of the ER stress marker proteins GRP78 and PDI and autophagy marker, beclin-1 (Fig. 7A). ER stress inhibition by TUDC and knockdown of IRE1 inhibited autophagy as indicated by reduced expression of beclin-1 (Fig. 7A, B). These results demonstrate that MCPIP-induced adipogenesis is mediated via ROS induction of ER stress that leads to autophagy.

MCPIP-induced adipogenesis involves p38 activation

ER stress is also known to lead to p38 activation that can cause autophagy [26, 27]. To test if p38 was activated during MCPIP-induced adipogenesis, p38 was immunoprecipitated from the cell lysate of MCPIP expressing 3T3-L1 preadipocytes. Immunoprecipitated p38 was incubated with activating transcription factor (ATF) 2, a known target of activated p38 [28], and phosphorylation of ATF2 was examined by immunoblot analysis. MCPIP expressing cells showed a time-dependent increase in ATF2 phosphorylation with maximal activation occurring on day 5 after transfection with MCPIP expression vector (Fig. 8A). Thus, MCPIP induces p38 activation during the later stages of MCPIP-induced adipogenesis.

To test if MCPIP-induced p38 activation is involved in MCPIP-induced adipogenesis, 3T3-L1 cells were treated with the p38 inhibitor SB203580 (Fig. 8B). Inhibition of p38 attenuated expression of the adipocyte markers adiponectin and LPL and reduced oil droplet

accumulation (Fig. 8C). These results showed that MCP-1-induced adipogenesis involves p38 activation.

Discussion

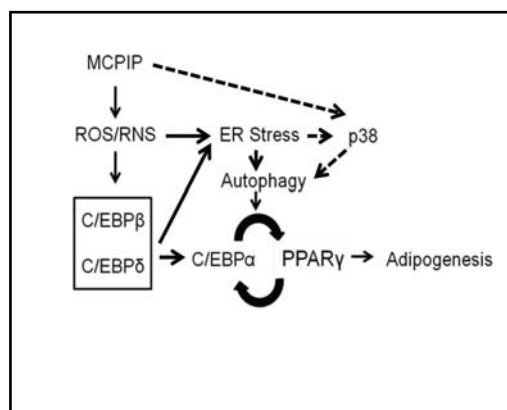
There is growing evidence for the involvement of low grade inflammation in the pathophysiological progression of obesity and type 2 diabetes [10]. MCP-1 is known to be produced in adipose tissue, and its involvement in adipogenesis has recently been demonstrated [13]. MCP-1 induced adipogenesis is mediated via the novel zinc-finger protein, MCPIP [13]. The results presented in this report elucidate the intermediate processes involved in MCPIP-induced adipogenesis and thus provide a molecular framework explaining how MCPIP might induce adipogenesis and further establishes MCPIP as a newly identified player in the adipogenic process.

NO plays an important role in inflammatory responses. Increased expression of iNOS is observed to be associated with obesity and type 2 diabetes and is thought to be an important player in adipose tissue development. This increased iNOS expression most likely occurs due to an increase in cytokine levels. In a tissue engineering model, inhibition of iNOS with aminoguanidine prevented MCP-1 induced adipose tissue formation [14]. Our finding that MCPIP-induced adipogenesis induces iNOS expression is consistent with these observations. NO production is known to induce C/EBP β expression via intracellular increases of cGMP that results in the induction of cAMP [29]. Thus, NO production is important for the early stages of adipogenesis. This is consistent with our observation that iNOS expression is elevated only during the early periods of MCPIP-induced adipogenesis.

Insulin has been found to be responsible for the induction of NADPH oxidase in preadipocytes [17]. The importance of insulin-induced ROS production during adipogenesis is further evidenced in a study where 3T3-L1 cells were treated with dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) but without insulin [16]. Adipogenesis in the 3T3-L1 cells treated with this incomplete cocktail was attenuated, while treatment of cells under the same conditions supplemented with hydrogen peroxide restored the adipogenic response. Some reports suggest that a decrease in ROS production is necessary for adipogenesis to occur [29]. This could partly be explained by the use of multiple reagents for the induction of adipogenesis. Dexamethasone has been shown to downregulate phox47 expression in THP-1 monocytes [30]. Induction of cAMP, a function of IBMX, inhibits parathyroid hormone and pertussis toxin induced oxidative bursts in osteoclasts [31]. However, the key ingredient needed to induce robust adipogenesis is insulin [17]. Our finding that the potent NADPH-oxidase inhibitor, apocynin, attenuated MCPIP-induced adipogenesis is consistent with these observations that demonstrate a role for NADPH oxidase mediated ROS production in adipogenic events. Interestingly, apocynin did not completely obliterate MCPIP-induced ROS production. Recently, apocynin was shown to induce iNOS expression in glial cells accompanied by an increase in oxidative stress [32]. This effect could explain the incomplete attenuation of ROS production observed in our study. Moreover, that apocynin did not completely obliterate MCPIP-induced ROS production and MCPIP-induced adipogenesis demonstrates the complexity of oxidative stress in cellular events including proliferation, death and differentiation.

ROS/RNS production can lead to an accumulation of misfolded proteins that results in ER stress [19, 20]. ER stress is associated with an increase in the expression of chaperone proteins including GRP78 and PDI [33]. If ER stress is prolonged, it can result in a series of signaling events known as the unfolded protein response. Recently, it was reported that DMI-induced adipogenesis was found to induce expression of ER chaperone proteins during the early to later stages of differentiation [21]. Our results show that knockdown of MCPIP attenuates ER stress that is involved in DMI-induced adipogenesis. Furthermore,

Fig. 9. Proposed model for MCPIP-induced adipogenesis. In 3T3-L1 preadipocytes, MCPIP expression induces ROS/RNS production that facilitates the expression of C/EBP β and C/EBP δ , transcription factors involved in the early stages of adipogenesis. MCPIP-induced ROS/RNS production further leads the induction of ER stress. MCPIP-induced ER stress results in the induction of autophagy. MCPIP-induced P38 activation is likely induced by ER stress and its activation can further lead to autophagy. However, MCPIP-induced autophagy results in increased expression of C/EBP α and PPAR γ and eventual adipogenesis.



the ER stress specific inhibitor TUDC [22] inhibited MCPIP-induced adipogenesis. MCPIP is known to induce ER stress in cardiomyoblasts [34]. These observations are consistent with our results demonstrating that GRP78 and PDI expression levels were elevated during the medial to later stages of MCPIP-induced adipogenesis. IRE1 is a main signaling component activated during UPR [33]. When UPR is activated, ATF6 induces the expression of X-box binding protein-1 (XBP-1) that is then cleaved by IRE1 [33]. XBP1-/- mouse embryonic fibroblasts have an impaired adipogenic response to DMI treatment [21]. Our finding that knockdown of IRE1 with siRNA attenuates transcript levels of C/EBP α and PPAR γ further validates the importance of UPR signaling in adipogenesis. Contrary to other observations and the data presented here, there is a previous report that suggests ER stress inhibits adipogenesis [35]. That study found that the GATA-specific inhibitor K-7174 attenuated DMI induced adipogenesis through the induction of ER stress as indicated by elevated expression of GRP78 and C/EBP homologous protein (CHOP). Furthermore, DMI induced adipogenesis was attenuated by treatment with the ER stress inducer thapsigargin that also resulted in increased expression of GRP78 and CHOP [35]. It is important to note that in that study, K-7174 induction of GRP78 and CHOP reached maxima 9 hr after treatment. Contrary to these findings, Basseri et al. found that when preadipocytes were treated with just DMI, CHOP decreased during the initial stages of adipogenesis while GRP78 and PDI expression levels were elevated [21]. However, during the later time points, CHOP expression increased. Taken together, these observations suggest that ER stress has biphasic control of the regulation of adipogenesis as it uses initial induction of certain chaperones such as GRP78 and PDI to facilitate C/EBP α and PPAR γ expression while CHOP signals the completion of adipogenesis and thus serves to turn off the adipogenic signaling processes [21]. These observations and the data in the present paper establish ER stress and the UPR as key regulators of adipogenesis.

A role for p38 in adipogenesis is becoming clear [28, 36, 37]. ER stress can lead to increased activation of p38 [26]. Recently, a study found that mice under high fat diet treated with the p38 inhibitor FR167653 had reduced weight gain as compared mice treated without the inhibitor [28]. These mice were found to have decreased mRNA levels of PPAR γ while mRNA levels of C/EBP β , C/EBP δ and C/EBP α remained unchanged. Our results are consistent with these observations as p38 activation occurred after the onset of MCPIP-induced ER stress. We also observed that MCPIP-induced adipogenesis was attenuated by the p38 inhibitor SB203580. This is consistent with reports showing that treatment with p38 inhibitors attenuated adipogenesis induced by DMI [28, 37] as DMI induced adipogenesis is mediated via MCPIP [28, 37]. However, while our data suggests that MCPIP-induced p38 activation occurs downstream of MCPIP-induced ER stress, further studies are needed to rule out the possibility that MCPIP-induced p38 activation occurs independent of ER stress.

ER stress-induced p38 activation is known to lead autophagy [26, 27]. Autophagy involves protein degradation that can play a critical role in differentiation as the differentiated state requires degradation of proteins for synthesis of a new set of proteins. Autophagy has been reported to be involved in differentiation in some other cellular contexts [38, 39]. Retinoic-acid induced differentiation of neuroblastoma N2a cells is inhibited by use of autophagy inhibitors 3' MA and LY294002 and via knockdown of the key autophagy regulator beclin-1 [39]. Autophagy has also been implicated in megakaryocytic differentiation [38]. Autophagy has been implicated in adipogenesis [24, 25]. In *atg5*^{-/-} mouse embryonic fibroblasts, adipogenesis was attenuated when cells were stimulated as compared to wild type fibroblasts [25]. That study also found that DMI induced adipogenesis in preadipocytes was attenuated when cells were treated with the autophagy inhibitor chloroquine [25]. Another study found adipose specific *atg7*^{-/-} mice were resistant to high-fat-diet induced obesity [24]. Our results provide strong support to the hypothesis that autophagy is involved in adipogenesis as MCP-1-induced adipogenesis displayed an increase in expression of the autophagy marker beclin-1 and pharmacological inhibition of autophagy attenuated MCP-1-induced adipogenesis. Beclin-1 has been proposed as a protein that can determine cellular fate in regards to autophagy, apoptosis and differentiation. B-cell leukemia/lymphoma 2 (Bcl-2) homologous proteins normally associated with the regulation of apoptosis, such as Bcl2 and Bcl-X_L, have been shown to interact with beclin-1 [40, 41]. These interactions have been demonstrated to be anti-autophagic and place beclin-1 as a key protein that can be involved in potential crosstalk between apoptosis and autophagy [40, 41]. Beclin-1 interaction with bcl-2 homologues occurs via its Bcl-2 homology domain 3 (BH3) [40]. It is plausible that beclin-1 interaction with bcl-2 or Bcl-X_L could promote apoptosis in the same way Bad, another BH3 protein, binds with bcl-2 or bcl-xl and inhibits their anti-apoptotic effects [40]. However, in a study showing that vitamin D3 induces autophagy mediated differentiation in HL-60 leukemia cells, chemical interference (ABT-737) of the vitamin D3 induced interaction between Bcl-X_L and beclin-1 resulted in increased apoptosis and had no effect on the observed induction of autophagy [42, 43]. In the HL-60 leukemia cells without vitamin D3 treatment, beclin-1 was at basal levels and disruption of the Bcl-X_L complex from beclin-1 also resulted in increased apoptosis [42, 43]. Thus, it appears that the expression level of beclin-1 is critical to the cellular decisions made in regards to apoptosis, autophagy and differentiation.

There is much evidence demonstrating a role for inflammation in obesity and type 2 diabetes [10, 11]. We previously reported that MCP-1 can induce adipogenesis via MCP-1 [13], but the molecular mechanism whereby MCP-1 could induce adipogenesis remained unknown. Here we provide a molecular basis whereby MCP-1 can induce adipogenesis. Our results demonstrate that MCP-1 induces ROS/RNS that results in the induction of the early adipogenesis transcription factors C/EBP β , C/EBP δ , and results in ER stress that leads to autophagy and the consequential induction of C/EBP α and PPAR γ that are involved in adipogenesis (Fig. 9). The observation that MCP-1 can induce these sequential events that lead to adipogenesis places MCP-1 as a key regulator of adipogenesis. Inflammation promotes obesity at least in part via MCP-1/CCR2 system. Development of obesity in high fat fed mice is attenuated by the absence of CCR2 [12]. However, the molecular mechanism underlying the role of CCR2 in the development of obesity was not known. Interaction of CCR2 with its ligand, MCP-1, is involved in the MCP-1 induction of MCP-1 [15]. The present results demonstrate how MCP-1 is critically important for adipogenesis, that causes increase in the number of adipocytes that contributes to obesity. Thus, MCP-1 and the molecular mechanisms it utilizes to induce adipogenesis provides new insights into how CCR2/MCP-1 interaction and inflammation contributes to obesity and have potential as therapeutic targets in the treatment and prevention of obesity and type 2 diabetes.

Acknowledgements

This work was supported by the National Institutes of Health [grant number HL-69458]. The authors confirm that there are no conflicts of interest.

References

- 1 Unger RH: Lipotoxic diseases. *Annu Rev Med* 2002;53:319-336.
- 2 Couillard C, Mauriege P, Imbeault P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Despres JP: Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. *Int J Obes Relat Metab Disord* 2000;24:782-788.
- 3 Darlington GJ, Ross SE, MacDougald OA: The role of c/ebp genes in adipocyte differentiation. *J Biol Chem* 1998;273:30057-30060.
- 4 Rosen ED, Spiegelman BM: Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 2000;16:145-171.
- 5 Yeh WC, Cao Z, Classon M, McKnight SL: Cascade regulation of terminal adipocyte differentiation by three members of the c/ebp family of leucine zipper proteins. *Genes Dev* 1995;9:168-181.
- 6 Schwarz EJ, Reginato MJ, Shao D, Krakow SL, Lazar MA: Retinoic acid blocks adipogenesis by inhibiting c/ebp β -mediated transcription. *Mol Cell Biol* 1997;17:1552-1561.
- 7 Elberg G, Gimble JM, Tsai SY: Modulation of the murine peroxisome proliferator-activated receptor γ 2 promoter activity by ccaat/enhancer-binding proteins. *J Biol Chem* 2000;275:27815-27822.
- 8 Wu Z, Rosen ED, Brun R, Hauser S, Adelman G, Troy AE, McKeon C, Darlington GJ, Spiegelman BM: Cross-regulation of c/ebp α and ppar γ controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell* 1999;3:151-158.
- 9 Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spiegelman BM: C/ebp α induces adipogenesis through ppargamma: A unified pathway. *Genes Dev* 2002;16:22-26.
- 10 Dandona P, Aljada A, Bandyopadhyay A: Inflammation: The link between insulin resistance, obesity and diabetes. *Trends Immunol* 2004;25:4-7.
- 11 Liang CP, Han S, Senokuchi T, Tall AR: The macrophage at the crossroads of insulin resistance and atherosclerosis. *Circ Res* 2007;100:1546-1555.
- 12 Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW, Jr: Ccr2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 2006;116:115-124.
- 13 Younce CW, Azfer A, Kolattukudy PE: Mcp-1 (monocyte chemoattractant protein-1)-induced protein, a recently identified zinc finger protein, induces adipogenesis in 3T3-L1 pre-adipocytes without peroxisome proliferator-activated receptor γ . *J Biol Chem* 2009;284:27620-27628.
- 14 Hemmrich K, Thomas GP, Abberton KM, Thompson EW, Rophael JA, Penington AJ, Morrison WA: Monocyte chemoattractant protein-1 and nitric oxide promote adipogenesis in a model that mimics obesity. *Obesity (Silver Spring)* 2007;15:2951-2957.
- 15 Zhou L, Azfer A, Niu J, Graham S, Choudhury M, Adamski FM, Younce C, Binkley PF, Kolattukudy PE: Monocyte chemoattractant protein-1 induces a novel transcription factor that causes cardiac myocyte apoptosis and ventricular dysfunction. *Circ Res* 2006;98:1177-1185.
- 16 Lee H, Lee YJ, Choi H, Ko EH, Kim JW: Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. *J Biol Chem* 2009;284:10601-10609.
- 17 Schroder K, Wandzioch K, Helmcke I, Brandes RP: Nox4 acts as a switch between differentiation and proliferation in preadipocytes. *Arterioscler Thromb Vasc Biol* 2009;29:239-245.
- 18 Yan H, Aziz E, Shillabeer G, Wong A, Shanghavi D, Kermouni A, Abdel-Hafez M, Lau DC: Nitric oxide promotes differentiation of rat white preadipocytes in culture. *J Lipid Res* 2002;43:2123-2129.
- 19 Gorlach A, Klappa P, Kietzmann T: The endoplasmic reticulum: Folding, calcium homeostasis, signaling, and redox control. *Antioxid Redox Signal* 2006;8:1391-1418.
- 20 Hsieh YH, Su IJ, Lei HY, Lai MD, Chang WW, Huang W: Differential endoplasmic reticulum stress signaling pathways mediated by inos. *Biochem Biophys Res Commun* 2007;359:643-648.
- 21 Basseri S, Lhotak S, Sharma AM, Austin RC: The chemical chaperone 4-phenylbutyrate inhibits adipogenesis by modulating the unfolded protein response. *J Lipid Res* 2009;50:2486-2501.

- 22 Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS: Chemical chaperones reduce er stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 2006;313:1137-1140.
- 23 Yorimitsu T, Klionsky DJ: Endoplasmic reticulum stress: A new pathway to induce autophagy. *Autophagy* 2007;3:160-162.
- 24 Zhang Y, Goldman S, Baerga R, Zhao Y, Komatsu M, Jin S: Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. *Proc Natl Acad Sci USA* 2009;106:19860-19865.
- 25 Baerga R, Zhang Y, Chen PH, Goldman S, Jin S: Targeted deletion of autophagy-related 5 (atg5) impairs adipogenesis in a cellular model and in mice. *Autophagy* 2009;5:1118-1130.
- 26 Nguyen DT, Kebache S, Fazel A, Wong HN, Jenna S, Emadali A, Lee EH, Bergeron JJ, Kaufman RJ, Larose L, Chevet E: Nck-dependent activation of extracellular signal-regulated kinase-1 and regulation of cell survival during endoplasmic reticulum stress. *Mol Biol Cell* 2004;15:4248-4260.
- 27 Kim DS, Kim JH, Lee GH, Kim HT, Lim JM, Chae SW, Chae HJ, Kim HR: P38 mitogen-activated protein kinase is involved in endoplasmic reticulum stress-induced cell death and autophagy in human gingival fibroblasts. *Biol Pharm Bull* 2010;33:545-549.
- 28 Maekawa T, Jin W, Ishii S: The role of atf-2 family transcription factors in adipocyte differentiation: Antiobesity effects of p38 inhibitors. *Mol Cell Biol* 2010;30:613-625.
- 29 Gummersbach C, Hemmrich K, Kroncke KD, Suschek CV, Fehsel K, Pallua N: New aspects of adipogenesis: Radicals and oxidative stress. *Differentiation* 2009;77:115-120.
- 30 Condino-Neto A, Whitney C, Newburger PE: Dexamethasone but not indomethacin inhibits human phagocyte nicotinamide adenine dinucleotide phosphate oxidase activity by down-regulating expression of genes encoding oxidase components. *J Immunol* 1998;161:4960-4967.
- 31 Berger CE, Horrocks BR, Datta HK: Camp-dependent inhibition is dominant in regulating superoxide production in the bone-resorbing osteoclasts. *J Endocrinol* 1998;158:311-318.
- 32 Riganti C, Costamagna C, Doublier S, Miraglia E, Polimeni M, Bosia A, Ghigo D: The nadph oxidase inhibitor apocynin induces nitric oxide synthesis via oxidative stress. *Toxicol Appl Pharmacol* 2008;228:277-285.
- 33 Zhang K, Kaufman RJ: From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008;454:455-462.
- 34 Younce CW, Kolattukudy PE: Mcp-1 causes cardiomyoblast death via autophagy resulting from er stress caused by oxidative stress generated by inducing a novel zinc-finger protein, mcpip. *Biochem J* 2010;426:43-53.
- 35 Shimada T, Hiramatsu N, Okamura M, Hayakawa K, Kasai A, Yao J, Kitamura M: Unexpected blockade of adipocyte differentiation by k-7174: Implication for endoplasmic reticulum stress. *Biochem Biophys Res Commun* 2007;363:355-360.
- 36 Takenouchi T, Takayama Y, Takezawa T: Co-treatment with dexamethasone and octanoate induces adipogenesis in 3t3-l1 cells. *Cell Biol Int* 2004;28:209-216.
- 37 Engelman JA, Lisanti MP, Scherer PE: Specific inhibitors of p38 mitogen-activated protein kinase block 3t3-l1 adipogenesis. *J Biol Chem* 1998;273:32111-32120.
- 38 Colosetti P, Puissant A, Robert G, Luciano F, Jacquel A, Gounon P, Cassuto JP, Auberger P: Autophagy is an important event for megakaryocytic differentiation of the chronic myelogenous leukemia k562 cell line. *Autophagy* 2009;5:1092-1098.
- 39 Zeng M, Zhou JN: Roles of autophagy and mtor signaling in neuronal differentiation of mouse neuroblastoma cells. *Cell Signal* 2008;20:659-665.
- 40 Oberstein A, Jeffrey PD, Shi Y: Crystal structure of the bcl-xl-beclin 1 peptide complex: Beclin 1 is a novel bh3-only protein. *J Biol Chem* 2007;282:13123-13132.
- 41 Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B: Bcl-2 antiapoptotic proteins inhibit beclin 1-dependent autophagy. *Cell* 2005;122:927-939.
- 42 Wang J, Lian H, Zhao Y, Kauss MA, Spindel S: Vitamin d3 induces autophagy of human myeloid leukemia cells. *J Biol Chem* 2008;283:25596-25605.
- 43 Wang J: Beclin 1 bridges autophagy, apoptosis and differentiation. *Autophagy* 2008;4:947-948.